The Effectiveness of Free Radical Initiator 1,1'-azobis(cycohexanecarbonitirile)

An Honors Thesis (HONRS 499)

by

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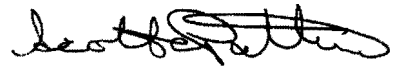

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Abstract

The project uses 1,1'-azobis(cyclohexanecarbonitrile) to mimic free radical damage done to human erythrocyte cell membranes. Free radical damage to the membrane can be lethal for the cell, but there might also be positive effects for the cell. Zinc is an essential element needed in all the body's cells, unfortunately channels or carries to transport zinc into the cell are only found in certain cells. One proposed mechanism to allow zinc uptake in to the cells is a partially oxidized membrane. This project uses the free radical initiator to controllably oxidize the membrane, and compare its effectiveness to other known free radical initiators.

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Introduction:

Diabetes, sickle cell anemia, and cancer all share one common link, an overly oxidized membrane. Numerous diseases can be linked to oxidative damage and recent research has lead to the exploration of oxidative damage in cells. Most of the research focuses on the negative impact of oxidation, although such oxidation is naturally occurring in cells. One positive result of oxidation is a mechanism to allow important materials into the cell. For ions such as sodium and potassium, a transporter into the cell has evolved because they are both needed in large amounts for proper functioning (*The World* 2000). Trace elements such a zinc are handled differently. Only certain cells in the body need a large amount of zinc, for example the axons of nerve cells (*Biochemistry* 2000). A special mechanism exists to bring zinc into the cells. Where zinc is not needed in large supply, a special channel or carrier would be a waste of the cell's energy for only a minimal amount (*Biochemistry* 2000). The membrane must have a way to transport the ion into the interior of the cell. Zinc, being a charged ion, is unable to pass through the normal phosophlipid bilayer without help of some sort, i.e. channels or carriers. One proposed mechanism, which allows zinc uptake in to cells, is a partially oxidized membrane (Ahen et al. 2004). The following experiment was designed to test and verify techniques to measure and study oxidized membranes. To study this system, a lipid-soluble free radical initiator was used to controllably oxidize the membrane and assay methods were used to quantitatively study the effects of the initiator.

Membranes are a complex system of molecules that comes together to provide a highly controlled environment for the cell. The constituents of the membrane give it strength, but are also part of its weakness to oxidative damage .The selectivity of the

membrane is an important feature, it allows for the passage of some molecules while not for others. Simple diffusion is a way for a small non-polar molecule to pass through the membrane without a carrier or channel for assistance (Wang et al. 2004). A biological membrane is composed mainly of chains of either saturated, monounsaturated, or polyunsaturated fatty acids (tail) linked to a phosphate backbone (head), Figure 1 (*The World* 2000). The arrangement of the phospholipids is tail to tail, forming a hydrophobic interior and a hydrophilic exterior.

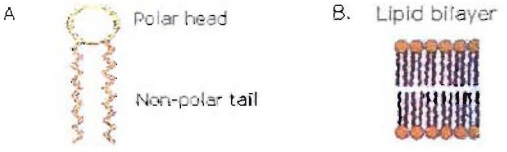

Figure 1
A. Representation of the phospholipid found in the membrane.
B. Representation of membrane structure, tail-to-tail lipid placement.

The two faces of the membrane are exposed to different environments. The intracellular side of the membrane has a distinctly different makeup of proteins and lipids then the extracellular side (*Biochemistry* 2004). The intracellular layer of human erythrocytes contain a greater percentage phosphatidylethanolamine (PE) and phosphatidylserine (PS) , while the extracellular layer contains a greater percentage of phosphotidylcholine (PC) and sphingomyelin (SP) (*Biochemistry* 2000).Figure 2 details the breakdown of composition in the human erythrocyte membrane and also the unique structure of the polar head groups. The membrane fatty acids can contain any where from zero to six double bonds, normally in a nonconjugated system (Curtain et al. 1980). The different components of the membrane make it more or less susceptible to oxidative damage.

Figure 2

A.

	PE	PS	PC	SP
Interior side of membrane	80%	100%	20%	15%
Exterior side of the membrane	20%	0%	80%	85%

B.

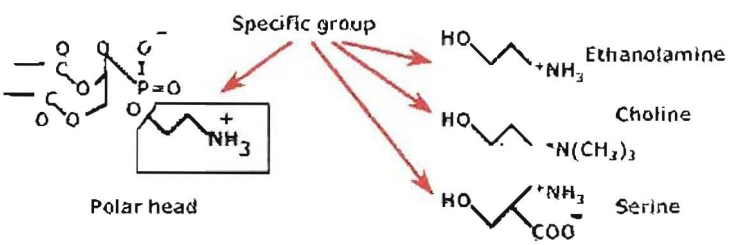

Figure 2

A. Details the percent compostition of different polar head groups in the human erythrocyte membrane.
B. The polar groups that make up the phospholipid head[4].

The lipid bilayer is constantly being bombarded with oxidation agents Some of the sources are internal and others are external. One known source for internal attack is the electron transport chain (ETC). The final step in the ETC is a four-electron reduction of O_2 to H_2O via one and two electron carriers, which are not always accurate (Yagi 1994). The carriers may not total reduce the oxygen generating incomplete oxygen species. One, two and three electron reductions form superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH) respectively (Porta et al. 2000). The formation of these species are collectively known as reactive oxygen molecules (ROS) (Yagi 1994) The byproduct of biological reactions, such as xanthine oxidase and amino acid oxidase, can be ROS's like H_2O_2 (*Biochemistry* 2000). The membrane is susceptible to ROS

attack via lipid preoxidation. The general mechanism for ROS attack is hydrogen abstraction at a bisallylic carbon forming a lipid radical, Figure 3 (Yagi 1994). This radical will react readily withoxygen diffusing through the membrane forming a lipid peroxyl radical, which can react the surrounding lipid chains (Molsen 1993). The primary product formed from lipid preoxidation is lipid hydroperoxide, which is a relatively stable form, and the reaction is terminated through reaction of antioxidants or proteins with the lipid radical (Yagi 1994).

Figure 3.

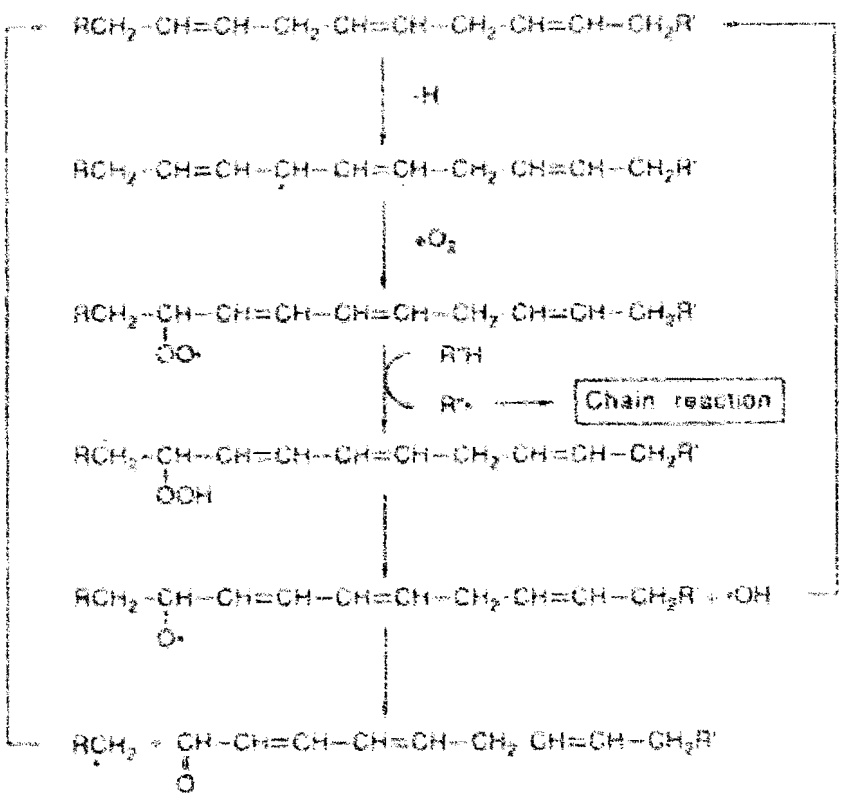

Figure 3. Scheme of free radical lipid peroxidaiton

Extensive oxidative damage to the membrane is lethal for the cell. As the oxidative process proceeds, the hydrophobicity of the membrane lessens and lysis occurs (*Biochemistry* 2000). A well-studied example of cell lysis is of red blood cells,

hemolysis. The oxidative effects on membrane lipids and peripheral and integral proteins have been documented in many cases. All cells are prone to such damage; therefore the membrane has incorporated mechanisms to fight the damage. The general defense mechanism involves cooperation between both the membrane and its surrounding environment. Within the membrane, free radical traps are one of the many types of mechanisms the cell has to stop damage. The surrounding environment, both intracellular and extracellular, contain enzymatic systems to regenerate radical traps and to reduce the destructive free radicals before they can do further damage (*The World* 2000). Also, damaged fatty acids are cleaved and replaced with normal fatty acids by enzymes. To prevent lysis of the cell, both the membrane and its surrounding environment work in sync to reverse the damage.

The membrane has antioxidants imbedded to help trap loose radicals. Vitamin E is a lipid soluble radical scavenger that helps to prevent further oxidation within the membrane. Its unique structure helps to trap single electrons one at a time. Once vitamin E has two electrons it is neutral and loses the scavenging ability (Porta et al. 2000). Enzymatic systems come into play to regenerate the oxidized form of vitamin E. Vitamin C is the water-soluble counterpart for vitamin E. Vitamin C acts by relieving vitamin E of two electrons and making it capable of scavenging radicals (Molsen 1993). Superoxide dismutase (SOD) is an enzyme that reacts with two superoxide molecules and forms oxygen and hydrogen peroxide (*Biochemistry* 2000). The hydrogen peroxide then reacts with catalase or peroxidases to form water and oxygen (Yagi 1994). Both the enzymatic and membrane systems play important parts in keeping the membrane functional.

Membrane lipid oxidation is a well-studied phenomenon because of its association with disease. Diabetes, sickle cell anemia and cancer are a few diseases believed to share a common link with membrane oxidative damage and correlate with erythrocyte oxidative damage. Two common ways to study oxidation of the erythrocyte membrane are measuring the hemoglobin concentration, indicating hemolysis, and measuring lipid peroxidation using thiobarbaturic acid (TBA) test. Studies use the hemolysis assay to estimate the overall damage done by oxidation, while using the TBA test to identify a mechanism for the damage. The focus of new research is to identify the differences in diseased membranes compared to normal membranes. Studies have shown that the "erythrocytes of diabetic patients have abnormal membrane properties" (Sato et al. 1989). A recent study found that abnormalities of the erythrocyte membrane in diabetic patients include a lower concentration of Vitamin E found in the membrane then in healthy patients (Yanagawa et al. 2001). The paper goes on to suggest that Vitamin E "uptake in erythrocyte membranes is significantly decreased" in diabetic patients and that the functional ability of Vitamin E is "due to impairment of this transfer mechanism, which may be associated with the pathogenesis of diabetes" (Yanagawa et al 2001). Another well studied disease is sickle cell anemia and the effects of an overly oxidized erythrocyte membrane. One study reports that the susceptibility of the membrane to damage is caused by an excessive production of H_2O_2, and the lack of catalase emzymes in the membrane to break H_2O_2 down (Das et al 1993). One cancer study related oxidative damage in erythrocytes to the insufficient defense systems of the membrane. SOD was one of the membranes defense mechanisms whose activity was recorded to be twenty-five percent less in cancer patients, along with a lack of Vitamin E (Pavri et al.

1983). The erythrocyte membrane is a dynamic system that has many contributing factors for its demise.

The basis for the exploration in the following project came from two papers by Yukio Sato et al (1989, 1995). Both papers involved controllable free radical-induced hemolysis of human erythrocytes, one paper used a water-soluble initiator and the other lipid-soluble initiator. Both papers studied protein and membrane aspects of the membrane to derive a mechanism for cell lysis. One specific protein studied in both papers was band three protein, an erythrocyte anion transporter. This protein is a critical component in the regulation of gylcolysis in the cell and a mediator of aged and/or damaged cell removal (Sato et al. 1989). Sato et al. findings correlate band three oxidation with membrane oxidation. The results of the papers show how different concentrations of the initiator with varying factors, temperature and time are related to the effectiveness of the initiator. Azo compounds 2,2'azobis(amidoinopropane) dihydrochloride (AAPH) and 2,2'-azobis(2,4-dimethylvaleronitrile) (ADVN) were the nonenzymatic water-soluble and lipid-soluble free radical initiators, respectively. The preceding compounds were formulated to induce oxidation in a controllable fashion. The following project used lipid soluble azo compound 1,1'-azobis(cyclohexanecarbonitrile) to induce oxidation of erythrocyte membrane lipid and compare the effectiveness of the initiator with the former experiments.

Materials and Methods:

Materials: 1,1'-asobid(cylohexanecarbonitrile) ABCC was obtained from Sigma-Aldrich Chemical Company. Whole blood was donated from Ball Memorial Hospital. All other chemicals were available through Ball State Chemistry Department.

Cell Preparation: The whole blood was washed in Hepes buffer [0.05M Hepes with 0.10 M NaCl, adjusted with KOH to a pH of 7.4] and spun down for 10 minutes at 5000 rpms and the supernatant aspirated off. The process was repeated three times to obtain pure erythrocytes (Sato et al 1989).

Hemolysis Assay: A ten percent erythrocyte suspension was subjected to 2mM, 5mM, or 7.8 mM ABCC for varying lengths of time at 37° C. After ten minutes of spinning at 13,000 rpm, analysis of the supernatant for hemoglobin was taken using UV-Vis at an absorbance of 540nm. For one-hundred percent hemolysis, a one to one-hundred dilution using erythrocytes and purified water was used and analyzed after spinning at 13,000 rpms. This procedure was adapted from papers by Yulio Sato et al (1989, 1990).

Lipid Peroxidation Assay: Ten percent erythrocyte suspension was subjected to 20 mM ABCC for varying lengths of time at 37° C. The samples were spun and washed successively with 5.0 mM, 2.5 mM, and 1.0 mM of NaH_2PO_4 hemolysis buffer pH 8.0 and spun for 15 minutes at 24,000 x **g**. The cells were resuspended with 500 μL and the TBA test was preformed. The procedure for the TBA test was adapted from Begona Manuel y Kennoy et al (2001).

TBA Test: One volume of resuspended membranes were added to double the volume of TBA reagent [2.08 mL 12 M HCl, 15 g Trichloroacetic acid, 0 375 g Thiobarbituric acid] and 50 µL BHT solution [0.02 g BHT and 5.0 mL isopropanol]. The suspension was heated in a hot water bath for 15 minutes then cooled in an ice bath, at 4 °C, for 15 minutes. 1 mL of n-butanol was used for the extraction of the organic layer. Analysis was done on the organic layer using UV-Vis at 532nm(Manuel y Kennoy et al. 2001).

Results

Hemolysis Assay: To controllably oxidize the membranes different amounts of ABCC were added to the erythrocytes. Two different methods of induction for the initiator were used with the second (alternate) method proving to be a better method. The temperature was kept at a constant 37 °C for the assays, to mimic the temperature of the human body Figure 4.

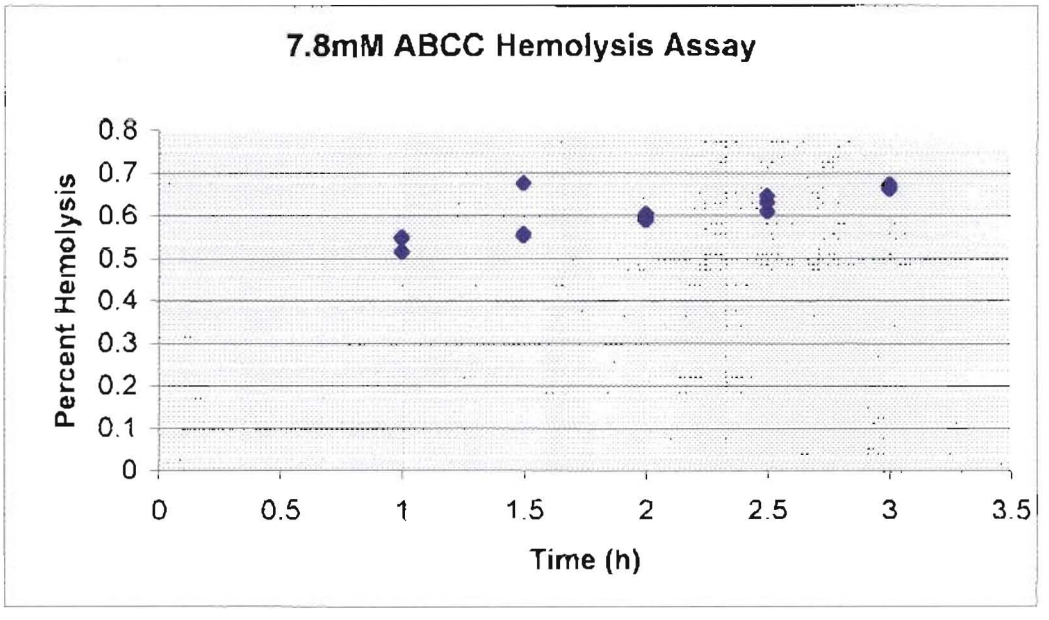

Figure 4 Time course of hemolysis of human erythrocytes using 7.8mM ABCC introduced to the cells through a coating on the flask, at 37 °C.

The first assay incorporated the initiator into the system in an ineffective way ABCC was first dissolved in cyclohexane, which proved to be an ineffective solvent. Next, methanol was added to the cyclohexane and ABCC solution to fully dissolve the remaining initiator. This solution was swirled in the bottom of the Erlenmeyer flask in order to coat the bottom of the flask with ABCC. A steady stream of nitrogen was used as an aid to evaporate off the alcohol. The cells were then added to the flask and swirled. After three hours, the maximum hemolysis was less then one percent, see figure 4. Only a small percent of the cells were affected by the initiator. 7.8 mM of ABCC was used because it comes in pellets and the mass of one small pellet gave the concentration of 7.8 mM in the erythrocyte suspension.

Figure 5.

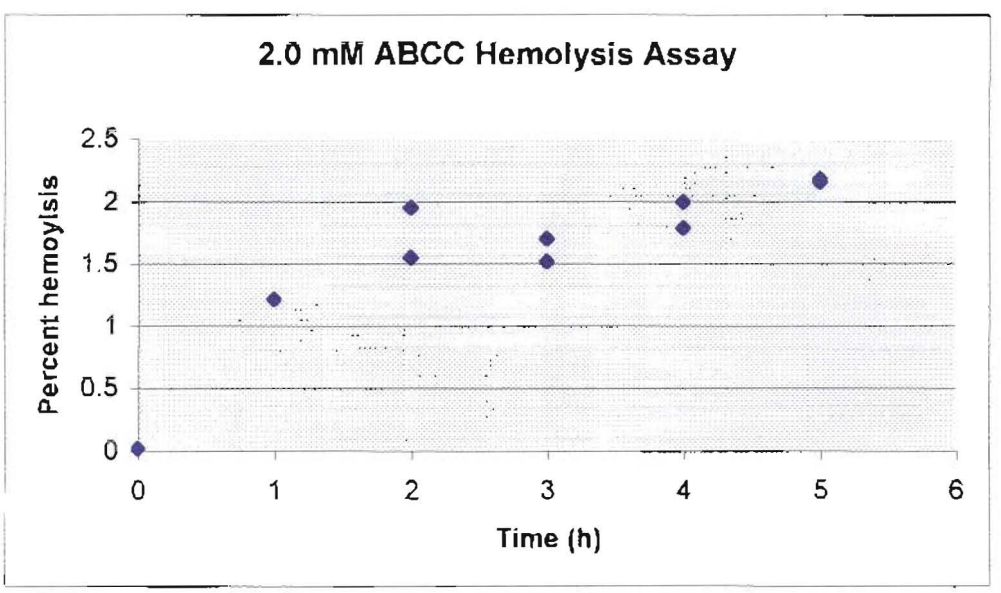

Figure 5. Time course hemolysis assay with 2.0mM ABCC introduced in an alternate fashion at 37 °C.

The second assay utilized an alternate method to introduce the initiator into the erythrocytes membranes This method used a small quantity of methanol and the ABCC compound. The initiator was dissolved in the methanol then poured into the red blood

cell suspension. The initiator precipitated out of the solution when added to the erythrocyte suspension. This method increased the effectiveness of the initiator because the total percent hemolysis for the assay increased, see Figure 5. The amount of initiator was cut in fourth while the percent hemolysis increased from below one percent to above two percent for this assay.

Figure 6

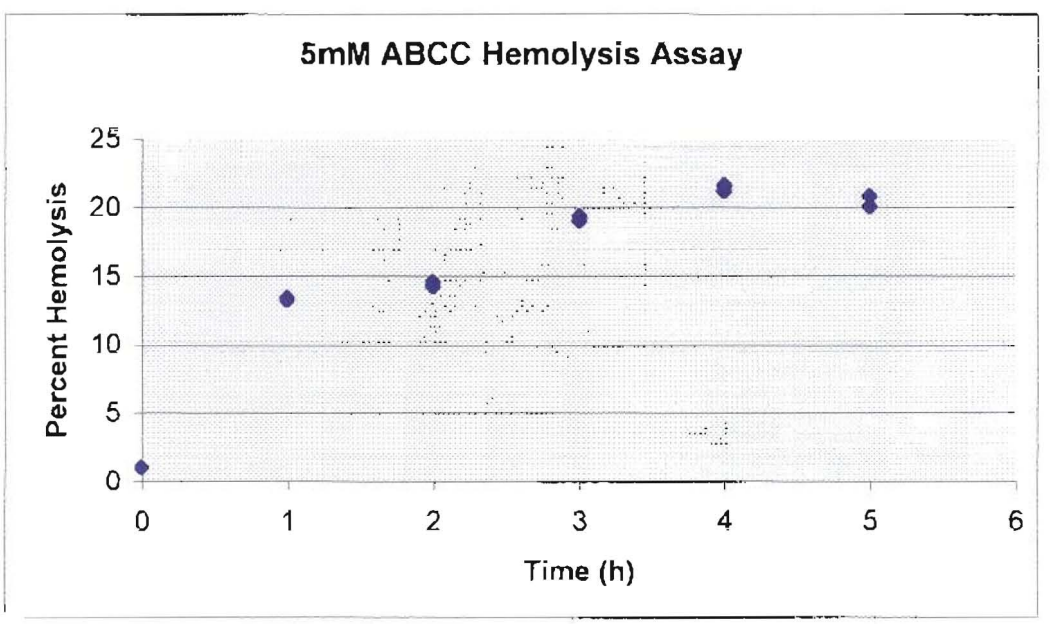

Figure 6. Time course hemolysis using 5mM ABCC introduced with the alternate method at 37 °C.

The third assay was done with the alternate way of introducing ABCC to the erythrocyte suspension. This assay shows a significant increase in the percent of hemolysis, see Figure 6. The concentration of ABCC was increased by 2 ½ times while the increase in hemolysis was near ten fold, see Table 1 for hemolysis assay results.

Table 1.

Assay	[ABCC]mM	Untreated cells percent hemolysis	Range of hemolysis
1	7.8	Not available	0.51 – 0.67
2	2	0.032	0.032 – 2.2
3	5	1.1	1.1 – 21.0

Table 1. Summarizes the parameters of the hemolysis assays.

In comparison to AAPH and ADVN, ABCC is a more effective initiator, because it requires a smaller concentration to cause the same amount to oxidative damage. Table 2 shows the comparitive values for AAPH, ADVN, and ABCC. The relative values for AAPH and ADVN are both 30mM concentration to produce the same amount of hemolysis in the erythrocytes.

Table 2.

Initiator	Concentration (mM)	Percent Hemoylsis
AAPH	30mM	~20 (after 6 hours)
ADVN	30mM	~20 (after 6 hours)
ABCC	5mM	22 (after 5 hours)

Table 2. Comparative values for free radical initiators AAPH, ADVN, and ABCC.

TBA Test:

Two experiments were used to prove the efficiency of the test. The first test was designed to measure the percent oxidation in untreated erythrocyte membranes. The

hemolysis assay was not performed on the untreated cells. The results for the untreated cells showed no oxidation, see Figure 7. The spectra for the trial TBA test shows no peak at 532nm, the wavelength for reading thiobarbaturic reactive species (TBARS).

Figure 7

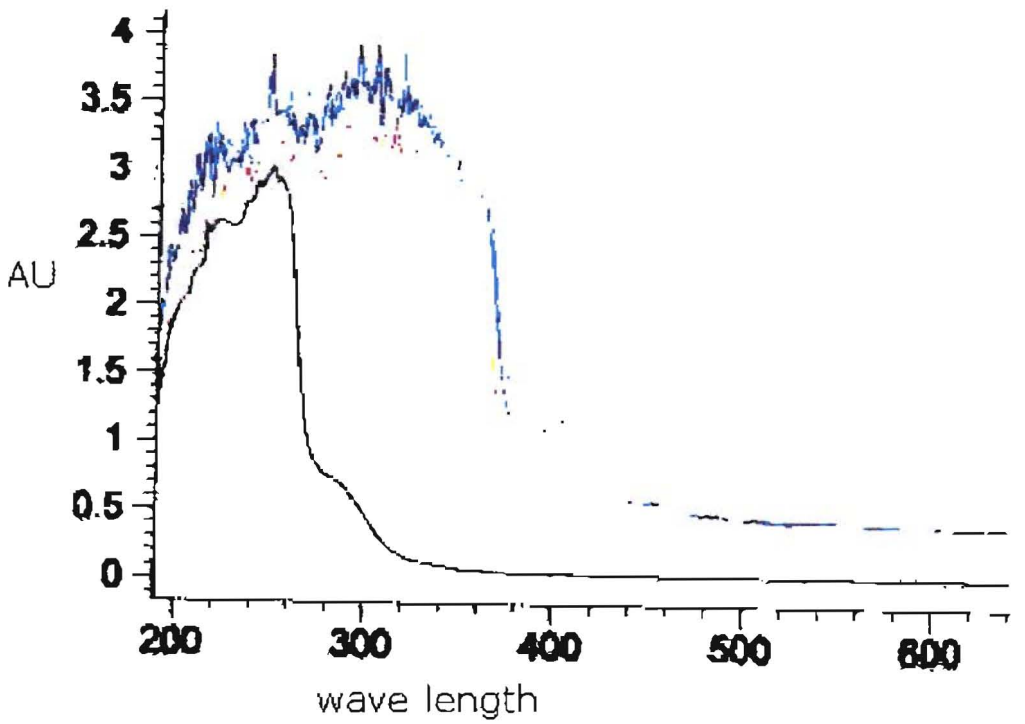

Figure 7. The spectra for TBA Assay 1. The black line represents butanol, the top line represents 500 μL of membranes, middle line 250 μL of membranes and the red line 100 μL of membranes.

The second of the experiments used a large amount of ABCC to initiate lipid oxidation. 20mM of ABCC over a time course of three hours brought significant cell lysis. No peak was found at 532nm for verification of the TBA test with any of the samples.

Figure 8.

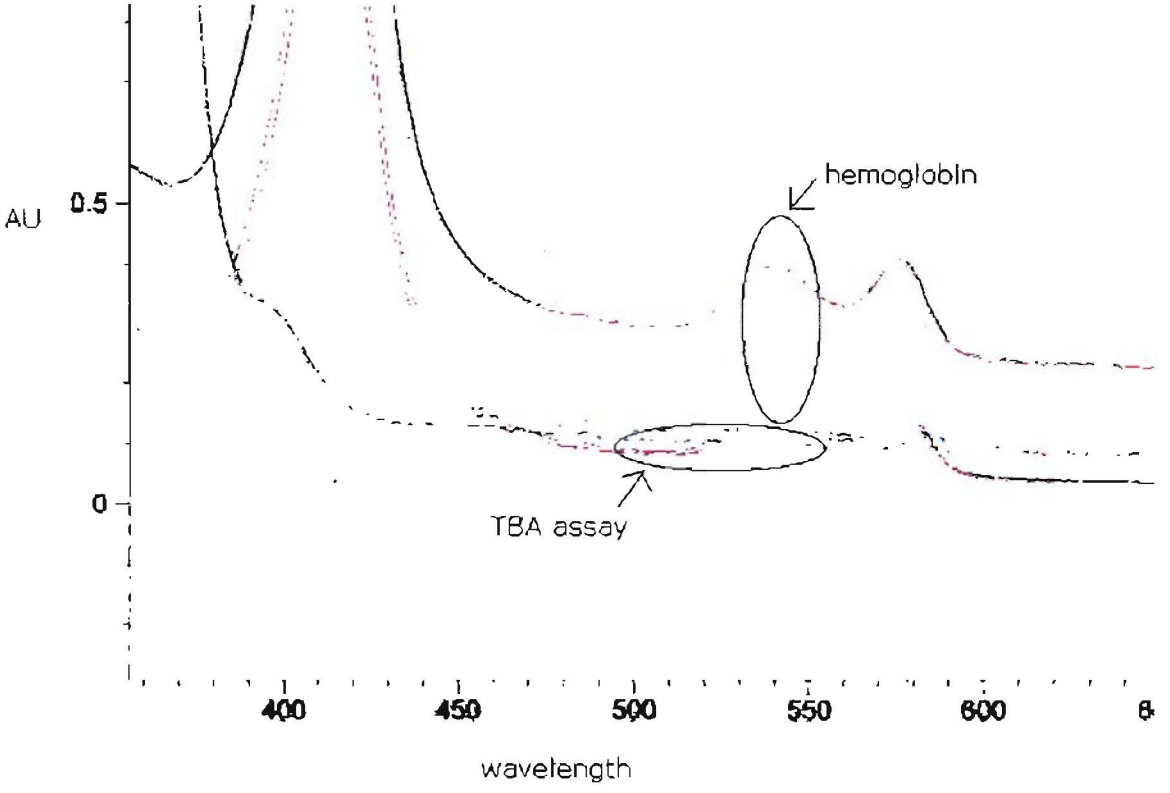

Figure 8. The spectra for TBA assay using 20mM of ABCC for 3 hours at 37 °C. The solid lines represent the hemolysis assay while the dashed lines represent the TBA test at 1-hour time intervals.

Discussion:

The goal of this project is to test the initiator compound for its effectiveness for controllable oxidizing erythrocyte membranes. Two test were used to qualitatively measure the effectiveness of ABCC. One indirect test for measuring its effectiveness is the hemolysis assay. The hemolysis assay is a easy way to test for extensive oxidative damage because competent erythrocytes have hemoglobin intact and incompetent erythrocytes, those with damage, will release the hemoglobin when the cell lyses. This is a test that gauges the amount the free hemoglobin found in the supernatant after the

cells have been centrifuged, using the UV-Vis spectra at 540 nm. The results of this assay are compared to known values for AAPH and ADVN. The direct test for the effectiveness of the initiator is the TBA test, by measuring TBARS-- oxidized lipids-- in untreated and treated cells. The TBA test would have been able to quantify the extent of oxidative damage in the erythrocyte membrane. Next comparison between the known values for AAPH and ADVN would have given direct comparison between the initiators The hemolysis is an indirect test because it does not test for the damage done to the lipids but rather the overall damage done to the cell.

Hemolysis Assay:

The first run for the assay shows only slight cell lysis, Figure 4. Many things were noted and corrected in an attempt to raise the level of oxidative damage done to the cell membrane. Cyclohexane was used as the first solvent to dissolve ABCC in. Methanol proved to be a better solvent for ABCC to be dissolved in and was used as the solvent throughout the rest of the assays. Because of the coating of the initiator on the flask, many cells were probably not exposed to ABCC. The method of introducing the initiator to the cells was changed based on the low hemolysis seen in the first trial. Figure 4 shows a percent hemolysis of below one percent; while the other assays show a greater increase after several hours, Table 1 summarized the range of hemolysis between the three assays. The procedure was modified from coating the flask with the initiator to adding the dissolved initiator directly to the cell suspension. Although the initiator precipitates out of solution, once added to the cell suspension, a greater number of membranes will come in contact with ABCC. Methanol is a compound that can pass through the membrane without assistance. Although some of the ABCC will precipitate out of solution, some

will travel with the methanol into the interior of the membrane where it can start the free radical chain reaction, Figure 3. Another change made in the procedure was the zero point reading for oxidative damage. A time zero point was not taken for the first assay. This is a very important part of the assay because it allows for a comparison between the untreated and treated cells. For the remainder of the assays, a time zero point was taken to gain a better understanding of the damage caused by the initiator.

The range of percent hemolysis for untreated cells ranged from less then 0.1 to 1.11 percent based on 4 trials, Table 1. The blood used for these assays came from two separate donations from Ball Memorial Hospital. Based on the results from the hemolysis assay, different batches of blood cells have varying levels of oxidative damage. One possible explanation is that many factors affect the condition of the membranes in erythrocytes. Age, sex, health condition, and many others contribute to the oxidative damage in the cell (Sato et al 1989). No factors or conditions were known about the blood cells used for this project.

The second and third hemolysis assays had higher oxidative damage done to the erythrocytes then the first assay. With a definite beginning point and using the alternate method of introduction of the initiator into the system, the quality of the initiator can be gauged. A comparison between the three different free radical imitators can be made. The two initiators Sato used were AAPH and ADVN, varying concentration and temperature. Both Sato et al. and the assays presented here used a temperature to mimic body temperature 37 °C (1989, 1995). Using a hot water bath to heat the erythrocyte suspension helped to keep the temperature constant. Using ABCC at a concentration of 5mM, Figure 6, shows the maximum percent hemolysis was 22 percent. The smallest

quantity used by Sato et al. was 10mM for the both AAPH and ADVN, at 37 °C. For 10mM of AAPH, the water-soluble initiator, the percent hemolysis after 6 hours was under 20 percent (Sato et al. 1989). For 10mM of ADVN, the lipid-soluble initiator, the percent hemolysis after 6 hours was under 10 percent(Sato et al. 1995). As the amount of initiator was increased, AAPH maximum percent hemolysis was just above 50 percent using 75mM and ADVN was just under 70 percent using 75mM (Sato et al. 1989, 1995). The results from Figures 5 and 6 show that ABCC is a more effective free radical initiator, requiring only 5mM to produce hemolysis around 22 percent. Rather then 30 mM of AAPH or ADVN to produce 20 percent hemolysis, see Table 2. Based on the results of the hemolysis assays, ABCC is an effective initiator and can cause free radical damage in the human erythrocyte membrane.

TBA Test:

The TBA test would have been a direct confirmation of the actual amount of lipid peroxidation found in the erythrocyte membrane. In the series of papers by Sato et al., two different methods were used. Both papers involved varying amounts of trichloroacetic acid, thiobarbituric acid, and extraction solvents. For the AAPH, the water-soluble initiator, the erythrocytes were diluted with a phosphate buffer to lyse the cells. Then half of the total solution was added to 12% trichloroacetic acid, to precipitate out the proteins. After centrifugation, the supernatant was added to 0.7% TBA and cooked in a boiling water bath for 15 minutes. The test was read at 535nm (Sato et al. 1989). The procedure for the lipid-soluble initiator, ADVN, involved different reagents and time. Instead of 12% trichloroacetic acid, a solution of reagents was used to precipitate out reagents and start the TBA test reaction. This solution of reagents included

8.1% sodium dodecyl sulfate, 20% acetic acid, and 0.8 % TBA (Sato et al.1995). The temperature the test was cooked in dropped 5 degrees, from 100 °C to 95 °C, but the time increased from 15 minutes to 60 minutes (Sato et al. 1989, 1995). The organic layer was extracted with butanol and pyridine and read at a 532nm (Sato et al. 1995). The procedure for TBA test presented in this project followed more closely with the lipid-soluble initiator procedure. The procedure was adapted from a recent paper published in *American Society for Nutritional Sciences* (Manuel y Keenoy et al. 2001). The TBA reagent was comprised of HCl, trichoroacetic acid, and TBA; along with a BHT solution to keep side reactions from forming. The time and temperature portion of the procedure followed the water-soluble procedure with 15 minutes of cooking in a boiling water bath, then cooled in a ice water bath for 15 minutes before extraction of the organic layer. The test was read at 532nm, but Figures 7 and 8 show no peaks at either wavelength of 532 nm or 535nm.

This project will hopefully help the research currently going on at Ball State University. By having procedure that can controllably oxidize the membrane, new experiments can be formulated to find the missing link as to zinc uptake into cells. A major revision will have to be done in order to gather information as to the estimation of lipid peroxidation. Overall, the procedure for free radical induced lipid oxidation by lipid soluble ABCC has been fine tuned to controllably oxidize the human erythrocyte membrane. With minor adjustments, a direct test for the estimation of lipid peroxidation can be found using ABCC and compared to the values of lipid peroxidaiton for AAPH and ADVN.

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